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Antigen detection using microelectrode array microchips

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Abstract

Procedures and results are described for multiplexed immunochemical assays using semiconductor microchips. The microchips used here are miniaturized arrays of individually addressable microelectrodes controlled by active CMOS circuitry. Electrode densities exceed 1000 per cm^2. The array chips are coated with a porous reaction layer material to provide a 'biofriendly' milieu overlaying the electrode array. Biotin is linked covalently to regions within the porous reaction layer proximate to selected microelectrodes. Covalent linkage is accomplished using reagents that are generated in situ by the microelectrodes. The covalent linkage of biotin within the porous reaction layer allowed traditional streptavidin (SA)-based immunoassay formats to be used on the biochips.

Biochips were used to develop multiplexed assay formats for biological entities over a wide size range — from small organic molecules to cells. Sandwich immunoassays were used for larger entities and competitive immunoassays for smaller molecules. Detection of analytes was accomplished using fluorophore-tagged antibodies and epifluorescent microscopy. Results from a broad range of analytes are presented.

Keywords: Biochip; Immunoassay; Biosensor; Silicon chip; Proteomic chip

1. Introduction

Miniaturized arrays of biomolecules on a variety of platforms have come into prominence recently. Much of the initial focus for these microarrays has been in the genomics arena, with an early emphasis on single nucleotide polymorphisms (SNPs) and genomic DNA detection/validation, functional genomics, and proteomics [1–7].

Microarrays have great potential for use in a variety of immunoassay formats. However, there are significant technical challenges in immobilizing antibodies, proteins or analytes in specific and selected sites while retaining the activity of the biological moiety. Most platforms examined to date utilize spotting techniques to deliver the antibody or protein. Glass slides are frequently used as a substrate. Glass surfaces have a long history of being problematic for assay development. Also, spotting techniques often require that a particular antibody or protein "be dried" as part of the preparation and storage of the biomaterial [8–11].

The biochip array platform produced by CombiMatrix has a wide range of applications, from DNA synthesis/diagnostics to immunochemical detection. CombiMatrix biochips are semiconductor devices that employ complimentary metal oxide semiconductor (CMOS) technology to create high-density arrays of microelectrodes with parallel addressing for selecting and controlling individual microelectrodes within the array. The array of microelectrodes is coated with a porous reaction layer material. Thickness and porosity of this material are carefully controlled. Biomolecules

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are synthesized or immobilized within this porous reaction layer.

Immunochemical assays are highly selective and are in current use for monitoring many chemical and biological molecules. The biochip array platform technology described here affords the opportunity for multiplexing numerous chemical and biological immunoassays on a single microchip. CombiMatrix has developed methods that enable immobilization of different biomolecules at different microelectrodes in an array of individually addressable microelectrodes [12,13]. These methods enable multiplexed immunochemical assays on a single biochip. This paper demonstrates that multiplexed immunochemical assays on electrode array biochips have the potential to detect analytes ranging from small molecules (saxitoxin (STX) and antibodies) to viral particles, spores and cells.

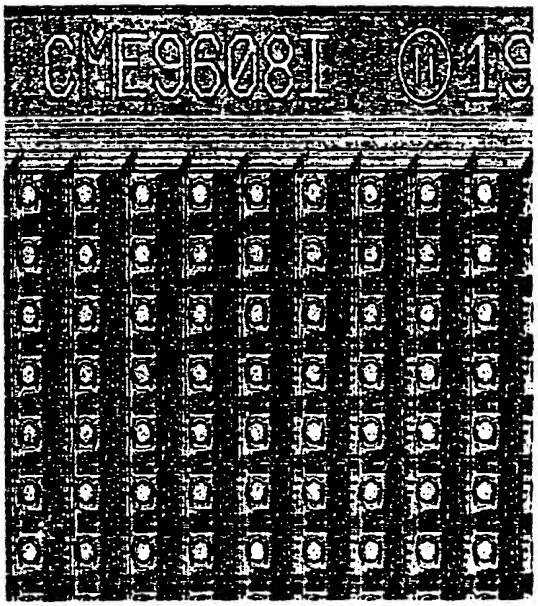

Fig. 1. A light photomicrograph of first generation CombiMatrix biochip (CME9608I).

2. Experimental

2.1. CME9608I chips

CombiMatrix produces CMOS semiconductor microchips that are high-density arrays of individually addressable microelectrodes. A white light photomicrograph of a portion of the first generation CombiMatrix electrode array biochip (CME9608I) is shown in Fig. 1. The CME9608I chip has 1024 microelectrodes that are each 100 μm in diameter. Each microelectrode in the array can be addressed independently using a 10-bit addressing scheme. Each microelectrode can be switched to one of four independent electrical channels. Quality control and state validation hardware is built into each microelectrode site. The CME9608I chip is controlled by custom hardware and software that was developed by CombiMatrix.

2.2. Porous reaction layer and reagent immobilization

The CombiMatrix biochips are coated with proprietary porous reaction layer material (Fig. 2). This material is ca. 1 μm thick. There are numerous advantages to immobilizing and synthesizing biomolecules within a thick porous layer. These include a much larger number of moieties per unit area, a three-dimensional geometry and the potential for a well hydrated and 'biofriendly' milieu.

Biotin was immobilized within the porous reaction layer at selected microelectrode sites via electrochemical methods that has been described previously [12]. Typically, the biochip was placed into a 1.8 ml solution of acetonitrile/methanol containing biotin–*N*-hydroxysuccinimide (b–NHS) and various electrolytes. For our electrochemical system, the reference was ground and a neighboring microelectrode served as the counter electrode.

Each biotin-labeled site can then conjugate with streptavidin (SA), which in turn can capture biotin-labeled proteins or antibodies. Biotin labeling is the basis for many immunochemical detection systems.

2.3. Reagents

Reagents, b–NHS, Texas Red–*N*-hydroxysuccinimide (TR–NHS), and fluorescein–*N*-hydroxysuccinimide (F–NHS) were purchased from Molecular Probes, Eugene, OR. Biotin–PEG–NHS (b–PEG–NHS) was secured from Shearwater Polymer, Birmingham, AL. SA, Texas Red-labeled streptavidin

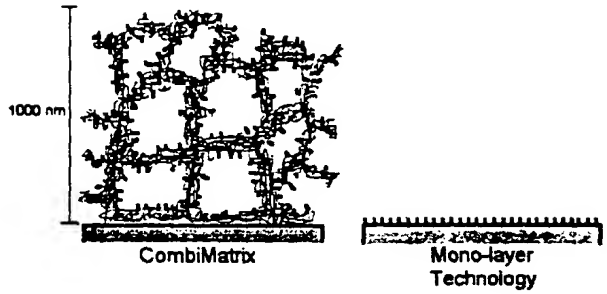

Fig. 2. A porous reaction layer is placed onto the CombiMatrix biochip. Reagents can be immobilized onto the porous layer.

(SA–TR), fluorescein-labeled streptavidin (SA–F), biotinylated–murine IgG, and TR-labeled-goat anti-mouse IgG are products of Vector Labs, Burlingame, CA. *Bacillus globigii* (BG) spores and goat sera containing anti-BG antibodies were obtained from Aberdeen Proving Grounds, MD. STX (diacetate) was purchased from Cal Biochem, San Diego, CA. The antibody used for this analyte was a rabbit-polyclonal, obtained from Monoclonal International, Sacramento, CA.

2.4. Procedures

Labeled anti-saxitoxin antibody was obtained as follows: rabbit serum containing polyclonal anti-saxitoxin antibody was obtained from Custom Monoclonals International, Sacramento, CA. IgG was purified using a protein-G column (Amersham Pharmacia); purified IgG was eluted at pH 4.5. The antibody was labeled with TR–NHS using the procedures outline for "antibody labeling" in the Molecular Devices Threshold (Sunnyvale, CA) Manual. TR–NHS reaction by-products were removed from the TR-labeled antibody using a Clontech spin-column.

The antibody used in all our BG detection experiments was derived from goat serum provided by Aberdeen Proving Ground. The polyclonal goat anti-BG antibody was obtained from the goat serum after purification and isolation of the IgG from a protein-G affinity column (Amersham Pharmacia). Approximately, 6 mg of IgG were obtained from 2 ml of serum. An amount of 1 mg of purified IgG was labeled with F–NHS as per procedures described above. Another milligram of the antibody was labeled with b–PEG–NHS (also tried b–DNP–NHS). A 0.5 mg antibody fraction was also labeled with TR–NHS and purified on a Clontech spin-column.

Saxitoxin-labeled streptavidin was prepared as follows [14]: streptavidin (Vector Labs, Burlingame, CA) was succinylated with succinic anhydride under basic conditions. The modified streptavidin was separated from the reaction by-products and salts using a 100 μl spin-column (Clontech, Palo Alto, CA). The sample was freeze-dried for storage. A fraction of the succinylated streptavidin was mixed with saxitoxin in phosphate buffer at neutral pH. Coupling of saxitoxin to the succinylated streptavidin was accomplished using a water-soluble carbodiimide. The sample was purified once more using a Clontech Chroma spin-10 column.

BG spores were tagged with TR label based upon the covalent linkage of TR to proteins on the spore surface containing free amino groups. This was accomplished in a manner similar to the labeling of proteins. However, a large excess of TR–NHS was used and the hydrolyzed (not reacted with protein) TR was removed by at least five washings with phosphate buffer, pH 7.0. The spore washing was accomplished by centrifugation of the spores in a 1.5 ml Eppendorf microtube and the supernatant was removed. More phosphate buffer is added, the spores vortexed, and the sample centrifuged. The process was repeated until the supernatant was clear.

Biotinlyation of the porous reaction layer was accomplished via an electrochemically mediated reaction involving a biotin–NHS ester and hydroxyl groups within the porous reaction layer [12]. The extent of biotinylation was determined by dipping a lower portion of the biochip in TR-labeled streptavidin and measuring the flourophore incorporation with an epifluorescent microscope. The biochip was

then washed and the entire biochip coated with unlabeled streptavidin.

A dilution series for saxitoxin on the CombiMatrix biochip was prepared as follows: a biochip was coated with a porous reaction layer as noted in earlier reports [12,13]. At this point, reagents for the incorporation of biotin–polyethyleneglycol (b–PEG) onto the porous reaction layer surface were added and the b–PEG was attached using electrochemically generated reagents. The difference in the present study was that the current to designated electrodes was set to varying time intervals. The current "on time" is directly proportional to the quantity of biotin moiety that is covalently attached to the porous reaction layer. Consequently, this affects the quantity of streptavidin–saxitoxin (SA–STX) that can be bound to the porous reaction layer under saturation conditions. The binding of TR-labeled anti-saxitoxin antibody (PAb) to the porous reaction layer-bound saxitoxin can provide a dilution series.

2.5. Immunoassays and analyte detection

The general assay procedure was as follows: a CombiMatrix biochip was coated as described above.

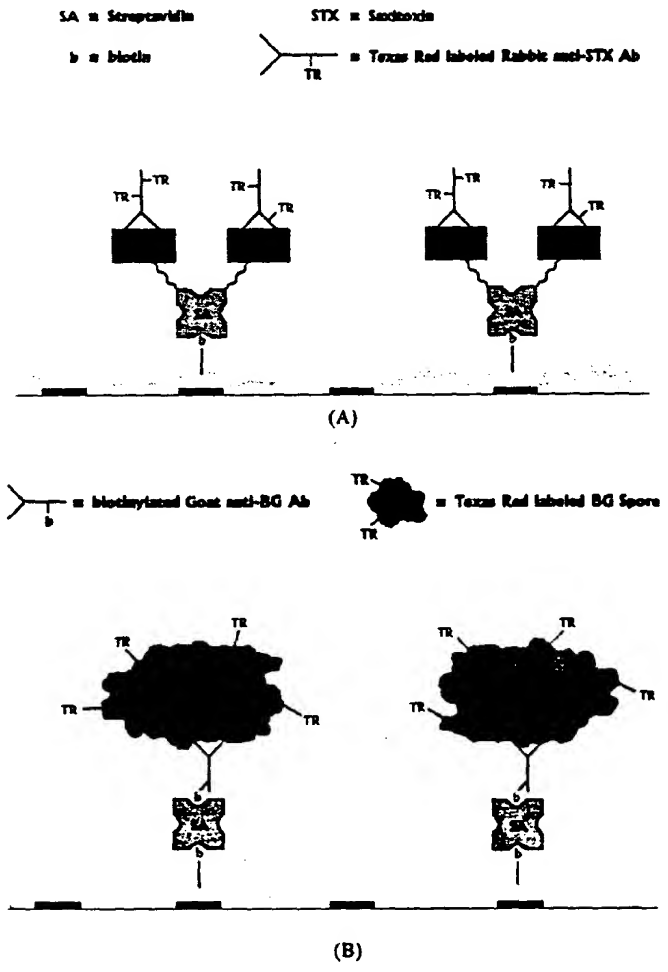

Fig. 3. (A) Competitive immunoassay scheme for saxitoxin. (B) Immunoassay scheme for TR-BG spores. (C) Immunosandwich assay scheme for BG spores. (D) Immunoassay for the detection of murine IgG on the CombiMatrix biochip.

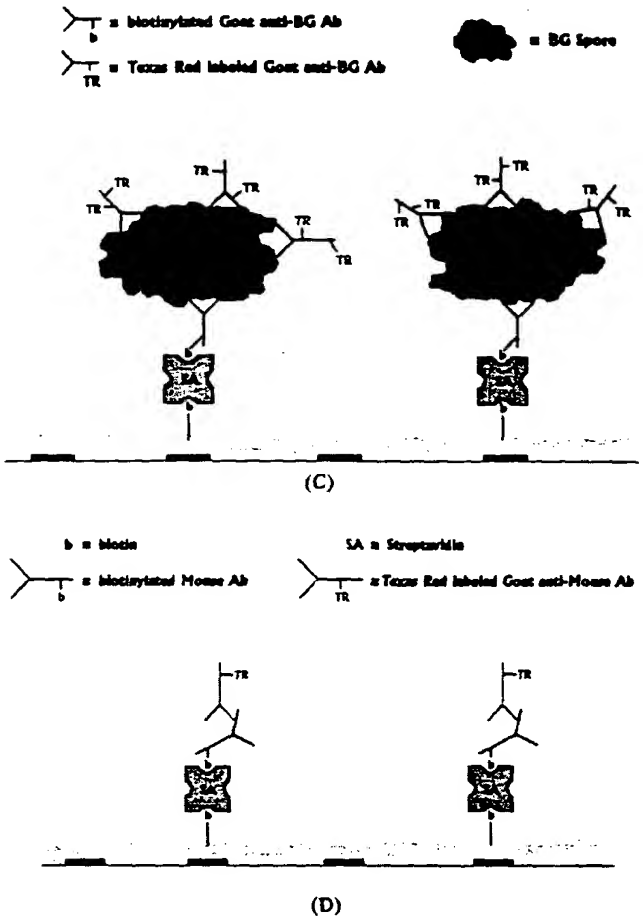

Fig. 3. (*Continued*).

After biotinylation, the biochip was soaked in a solution containing the SA–STX conjugate. The biochip was washed and then placed into a solution containing the TR-labeled-rabbit anti-saxitoxin antibody (the signal generator). After this incubation, the biochip was washed and the fluorescence measured.

For the case of saxitoxin, an indirect detection method is required. In this case, SA–STX conjugate is applied to a biotinylated biochip surface. The TR-labeled anti-STX antibody is added and allowed to incubate for 2 h. The excess antibody is then removed by washing of the biochip (Fig. 3A). For the maximum signal to occur, no analyte (free STX; analyte being tested) must be present in the solution containing the anti-STX antibody. The minimum signal would be expected if the anti-STX antibody solution contains enough free STX to saturate the antibody before the solution is applied to the porous reaction layer.

For the detection of TR-labeled spores (Fig. 3B), a site-specific b–PEG coated biochip was incubated with a solution containing streptavidin. The biochip was then extensively washed and placed into a solution containing b–PEG-anti-BG antibody. After rinsing the biochip, the TR-labeled spores were layered onto the biochip and placed into a humidifier for 1 h. The porous reaction layer was then washed, and the fluorescence was measured on the epifluorescent microscope (Olympus BX60 epifluorescence microscopy

system). Images were acquired and the integration time was controlled digitally using commercial video capture cards and software developed at CombiMatrix.

For the BG spores sandwich immunoassay (Fig. 3C), the initial procedures were the same as above. However, after the biochip had been soaked with biotinylated antibody, unlabeled spores were layered onto the biochip (1 h incubation). After the biochip was washed, TR-labeled-goat anti-BG antibody was added and allowed to soak for 1 h. The biochip was washed with phosphate buffer and water before fluorescence measurements were taken.

2.6. Interfering substances

The specificity and performance of any assay depends upon assay non-specific binding, assay specificity (no cross-reactions), and assay interference. To check for assay interference a number of substances need to be added to the assay mixture to determine if those materials add or detract from the assay performance.

To elucidate the effects of interfering substances, we investigated numerous moieties that could interfere with the saxitoxin and BG assays. In both cases, compounds were chosen that were similar in nature to the analytes that were being tested. For saxitoxin, we chose to use brevetoxin (another marine toxin), ricin (an extremely toxic glycoprotein), and digoxin (a naturally occurring host of compounds including digitalis). For BG spores, we chose *pombe* (yeast) and *Salmonella*.

3. Results and discussion

3.1. IgG immunoassay

One of the more simple immunoassays that can be undertaken is the detection of murine IgG. In this case, biotinylated murine IgG is bound to the porous reaction layer and the bound antibody is detected with TR-labeled-goat anti-mouse IgG. Fig. 3D shows this assay in pictorial form.

A CombiMatrix biochip was prepared such that the current applied to specific sectors of the electrode varied, consequently, varying the quantity of biotin layered onto the porous reaction layer directly over these electrodes. The quantity (concentration) of biotin at each electrode would then affect the quantity of bound streptavidin. Hence, the ultimate effect would be the amount of murine IgG that could be bound to the porous reaction layer. Such a dilution curve for the labeling of a biochip is shown in Fig. 4. The data was

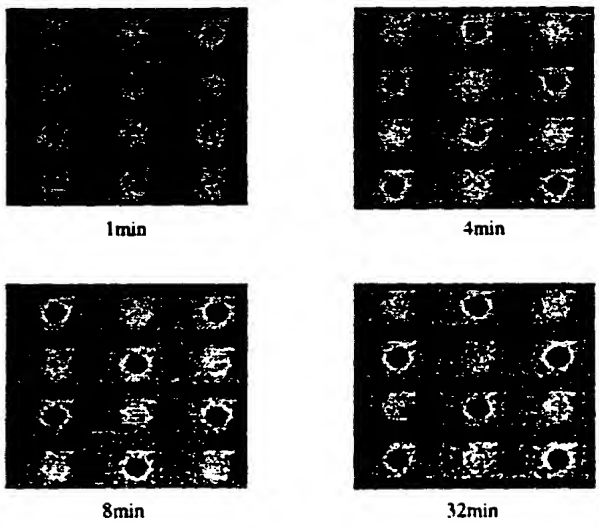

Fig. 4. Murine IgG dilution series. Sections of a single biochip are shown as a function of the current on time. Biotin is layered in a checkerboard pattern on the porous layer above the microelectrode.

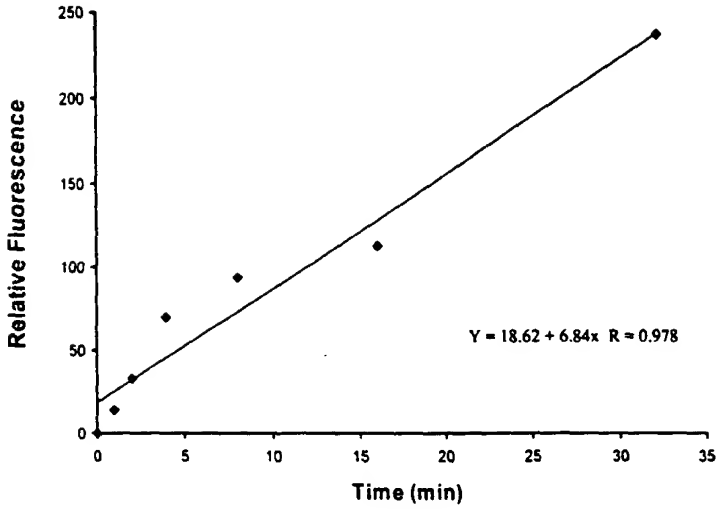

Fig. 5. Murine IgG dilution curve.

graphed and presented in Fig. 5. Note that the intensity increases with time in a linear fashion. This indicates that we do have murine IgG bound to the biochip surface and it can be detected with a secondary antibody.

3.2. The saxitoxin assay

Assay format for saxitoxin was followed as described above (Fig. 3A). The porous reaction layer was biotinlyated in a checkerboard pattern above selected electrodes and the current was varied so that a dilution curve could be obtained (as with case of IgG detection). After the biotinlyation has been accomplished, the biochip is soaked in SA–STX, washed and placed into a solution containing TR-labeled-rabbit anti-STX antibodies. The CombiMatrix biochip is then washed and the fluorescence measured. The results are shown in Fig. 6. The curve is linear for the most part, except at very high times (over 15 min). This could result from fluorescence quenching due to fluorophores being in close proximity. This would not be a problem if an alternative mode of detection were used.

To check for possible assay-interfering substances, we modified the assay such that the antibody was pre-incubated with the interfering substance (at 1 mg/ml) before the solution was spotted onto a section of the biochip containing bound SA–STX. We tested digitoxin, brevetoxin, and ricin in our assays. None of these materials significantly interfered with the saxitoxin assay.

3.3. Bacillus globigii assay

The simple method for the determination of whether spores can be detected on the biochip was to use TR–BG spores. In this way, only a single biotinylated antibody needs to be used. The antibody is layered onto the biochip and acts like an anchor for the spores.

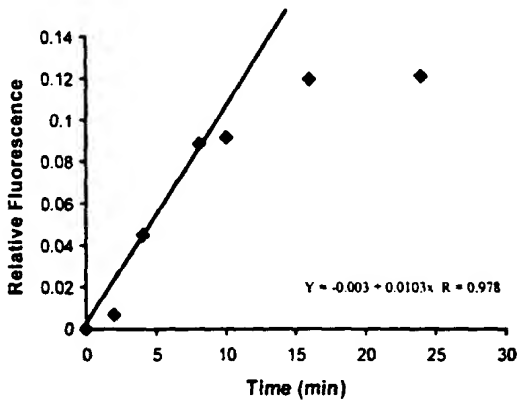

Fig. 6. Saxitoxin dilution curve.

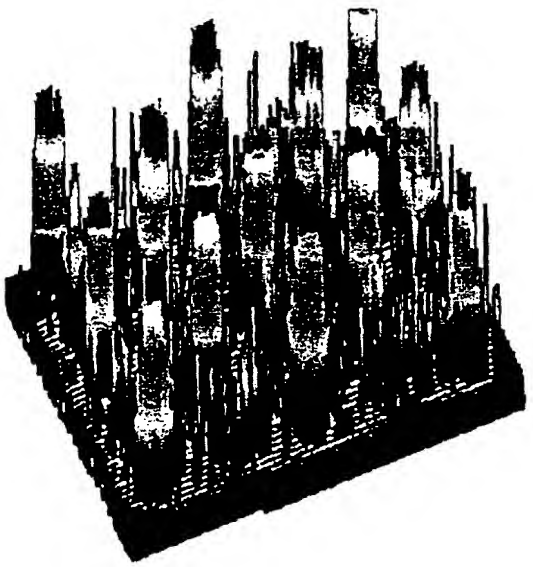

Fig. 7. Fluorescence micrograph for the capture of TR–BG on the CombiMatrix biochip.

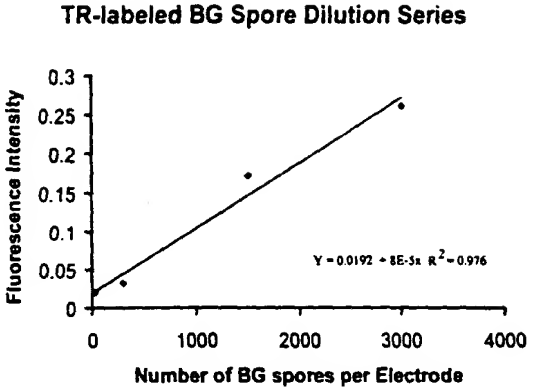

Fig. 8. Dilution series for TR–BG.

The spores are then layered on, allowed to incubate and the excess, unbound spores removed, with gentle washing. For future use, microfluidics may be an alternative to layering. Results can be seen in Fig. 7 for the capture of TR–BG on a uniform biotinlyated biochip containing SA and biotinylated goat anti-BG PAb. Note that the checkerboard pattern relating to the electrodes that were turned on.

A BG detection curve (loading study) was prepared based upon the binding of TR–BG spores to the biotinylated porous reaction layer. Diluted TR–BG spores (in 0.5 μl quantities) were placed over sections of the biochip and allowed to bind. The biochip was washed and the fluorescence intensity measured. The approximate quantity of spores bound to the porous reaction layer was calculated based upon the solution concentration of the spores and the relative area of electrodes (%) versus the entire biochip surface. The dilution results are shown in Fig. 8. This appears to be a linear plot with the limit of detection at about 50 spores.

A more realistic assay for BG spores would be to use non-labeled spores and one antibody to capture the spore while a second, TR-labeled antibody would be used to detect the spore. This would be called a sandwich assay as depicted in Fig. 3C. The only drawback under current conditions is that we have only a single type of antibody (PAb) available targeting identical BG-epitopes. A more practical solution would be to obtain a MAb for the BG spores while using the TR–PAb for detection.

However, for the current immunosandwich assay, we only had the one PAb available, so one portion of the Ab sample we biotinylated while the second portion was TR-labeled. In order to avoid competition for the same epitopes by these labeled antibodies, we used a sequential layering sequence. After the biotin and streptavidin had been placed on to the biochip, the biochip was soaked in a solution of biotinylated goat anti-BG antibody for at least 1 h. Following this, the biochip was extensively washed and BG spores were layered onto the biochip in a humidifier. After about 1 h, the biochip was washed and TR-labeled-goat anti-BG was layered on the biochip. After a 1 h incubation, the biochip was rinsed and fluorescence reading taken. The results are shown in Fig. 9. The sandwich assay does provide remarkable results. However, the assay performance could be improved if a biotinylated monoclonal antibody were used for the capture of the BG spores.

A BG standard detection curve was also developed using the assay procedures given above. The results are shown in Fig. 9. The procedure used for the calculation of the number of BG spores per spot is the same as that used for TR–BG. Note the near linear response over the range 0–1500 spores. The limit of detection (LOD) for this curve is approximately 100 spores. Again, the

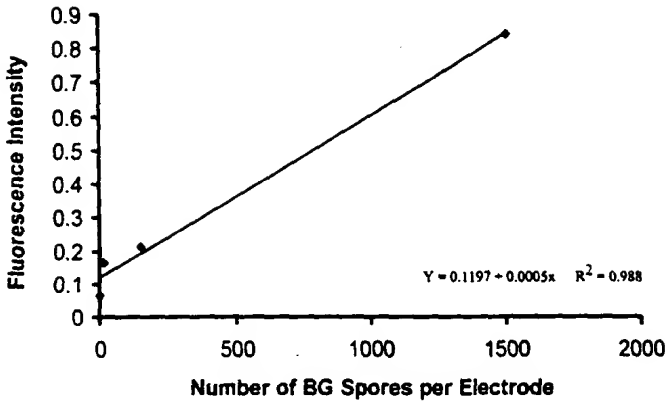

Fig. 9. Standard curve for BG spores using an immunosandwich assay.

assay performance could be improved if a monoclonal antibody were used to capture the spores.

For this assay, formaldehyde treated *Salmonella* and *pombe* (yeast) were used (at 1 mg/ml) to ascertain whether another cell system could interfere with the performance of the BG assay. In all experiments, it would appear that the performance of this assay is unhindered by the cells added to this mixture.

4. Conclusion

The CombiMatrix biochips have been used in this study to develop methods and protocols for implementation of multiplexed immunochemical assays on microarrays. Both competitive and sandwich immunoassay formats have been explored and have shown to be effective. The ability to implement several immunoassay formats simultaneously on the same microarray platform allows spatial multiplexing a large number of immunochemical assays on a single biochip. The versatility of this microarray platform was demonstrated by selective capture of very large analytes (*B. globigii* spores) and small protein toxins (saxitoxin). Two very different immunochemical assay formats were used: a competitive format in the case of saxitoxin and a sandwich format in the case of *B. globigii* spores.

Immobilization of immunochemical reagents at selected locations on CombiMatrix biochips is accomplished in a facile manner by using electrochemically generated reagents. These reagents are used in this study to immobilize biotin within a porous reaction layer proximate to individually addressed electrodes. Various dilution series in biotin can be made by changing the duration of the electrochemical modification step at different electrodes. After tagging sites with biotin, immunochemical reagents can be immobilized at these sites using conventional techniques. In this manner, large numbers of spatially multiplexed immunoassays can be presented on a single array biochip. These devices have the potential to discriminate between thousands of unique biological entities — all on microarray chip with a total area of less than 1 cm^2.

Multiplexed protein-based assays in a microchip format have a plethora of uses ranging from diagnostics to drug discovery. Proteomics, for example, is one emerging discipline where microarrays can have an enormous impact. Proteomic applications of arrayed biochips include: (i) monitoring levels of biomarkers in patients as a function of drugs and dosage administered to those patients; (ii) determining changes in protein expression levels in a cell as a function of genetic alteration; (iii) screening small molecule drug candidates; (iv) discovery and validation of novel drug discovery targets; (v) evaluating protein–protein interactions and; (vi) monitoring post-translational modification of proteins. The power and potential of arrays of microelectrodes for biochemical applications lay in the versatility and range of applications that can be developed on a single common hardware platform.

Acknowledgements

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